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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N A2

(11) International Publication Number: WO 91/06626

(43) International Publication Date: 16 May 1991 (16.05.91)

(21) International Application Number: PCT/US90/06128

(22) International Filing Date: 23 October 1990 (23.10.90)

(30) Priority data:

425,803	23 October 1989 (23.10.89)	US
502,272	29 March 1990 (29.03.90)	US
559,958	30 July 1990 (30.07.90)	US

(60) Parent Application or Grant
(63) Related by Continuation
US 559,958 (CIP)
Filed on 30 July 1990 (30.07.90)

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(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent), US.

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: TRIPLE HELIX FORMATION IN OLIGONUCLEOTIDE THERAPY

(57) Abstract

Oligonucleotides having tandem sequences of inverted polarity, i.e., oligonucleotides comprising regions of the formula: 3'-----5'--C--5'-----3' or 5'-----3'--C--3'-----5', wherein -C- symbolizes any method of coupling the nucleotide sequence of opposite polarity, are useful for forming an extended triple helix with a double-helical nucleotide duplex. Single or mixed motif oligomers may be used. The inverted polarity also stabilizes the single-strand oligonucleotides to exonuclease degradation.

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TRIPLE HELIX FORMATION IN OLIGONUCLEOTIDE THERAPY

Technical Field

The invention is directed to modes of therapy which utilize oligomers designed to form triple helices with duplex DNA. More specifically, the invention concerns provision of pharmaceutical compositions containing oligonucleotides which target the major grove of the DNA duplex.

The invention is also directed to oligonucleotides having tandem sequences of inverted polarity, which are useful for forming triple helices with double-stranded duplex DNA. The inverted polarity oligonucleotides may be stabilized by this inversion which presents an unnatural terminus or internal linkage, thereby avoiding potential damage by nucleases.

Background Art

The rules which govern the association of single-stranded oligonucleotides with DNA duplexes to form triple-helical complexes have been recently described. At present, there are two recognized motifs for effecting triple helix formation. The older of these, commonly referred to as the "CT" motif, provides for a single-stranded oligomer containing, in its essential recognition portions, pyrimidine-based sequences which will result in T-A-T and C-G-C^+ based triplets across the three associated chains of the resulting triple helix. This system is effective when there are long stretches in the duplex wherein one of the two strands contains only, or mostly, purine base

residues. The pyrimidine-containing oligomer will associate itself with the duplex in such a fashion that the polarity of the oligomer runs parallel to the strand of the duplex which contains the purine-rich target. The appropriate pyrimidine/purine associations are the same as those known in complementarity, i.e., in the oligomer T will be provided for recognition of an AT pair; C will be provided for the recognition of a GC pair. In summary, for formation of a triple helix which employs the CT mode, the oligomer will be designed to provide base "complementarity" to a purine-rich region of a single strand of the duplex in a parallel orientation to that strand. This mode of formation of triple helices was summarized by Moser, H.E., and Dervan, P.B., in Science (1987) 238:645-650.

An alternate motif, called the "GT" motif, was recognized more recently. In an article by Cooney, M., et al., Science (1988) 241:456-459, a purine-rich oligomer, containing a multiplicity of G residues, was reported to form a triplex with a DNA duplex which was rich in GC pairs and wherein the majority of the purine residues were located on a single strand of the targeted duplex. This mode of association results in G-G-C triplets across the three strands in the triplex and A-A-T triplets were suggested, although not demonstrated. In this mode, again, the purine residues need to be concentrated on one strand of the duplex; however, the orientation of the oligomer with respect to the target duplex is reversed from that in the CT mode described above--i.e., the oligomer will be oriented in an anti-parallel direction to the purine-rich strand of the duplex. The availability of the foregoing two motifs for triple helix formation offers expanded possibilities for the design of oligomers which are capable of triplex formation. It should be said, initially, that in all

instances, a concentration of purine residues along a portion of a single strand of the targeted duplex is required. Nevertheless, it is advantageous to have available a repertoire of strategies for targeting duplexes which permits advantage to be taken of additional factors which may influence the stability of the resulting complex or the suitability of the administered oligomer.

There are at least four major basic strategies for triple helix formation disclosed below. Two of these employ oligomers which contain inverted polarity. Thus, the invention provides oligonucleotides which have inverted polarities for at least two regions of the oligonucleotide. By "inverted polarity" is meant that the oligonucleotide contains tandem sequences which have opposite polarity, i.e., one having polarity 5'→3' followed by another with polarity 3'→5', or vice versa. This implies that these sequences are joined by linkages which can be thought of as effectively a 3'-3' internucleotide junction, (however the linkage is accomplished), or effectively a 5'-5' internucleotide junction. Such oligomers have been suggested as by-products of reactions to obtain cyclic oligonucleotides by Capobianco, M.L., et al., Nucleic Acids Res (1990) 18:2661-2669. Compositions of "parallel-stranded DNA" designed to form hairpins secured with AT linkages using either a 3'-3' inversion or a 5'-5' inversion have been synthesized by van de Sande, J.H., et al., Science (1988) 241:551-557. In addition, triple helix formation using an oligomer which contains an effective 3'-3' linkage has been described by Horne, D.A. and Dervan, P.B., J Am Chem Soc (1990) 112:2435-2437.

Disclosure of the Invention

The design of oligonucleotide sequences to target double-stranded duplex DNA can employ one or both of the above-described binding motifs, and may or may not employ oligonucleotides with inverted polarity, depending on the nature of the target duplex and other conditions characterizing the particular circumstance of administration of the oligomer.

Four basic situations can be envisioned. First, in a very simple case, understood in the art, the binding motif will be consistent along a standard oligomer of a single polarity. In this case, only a single strand of the duplex can be targeted, and while more than one purine-rich region along this strand can be engaged in triplex formation with oligomers of this type, such plurality of regions may be interrupted by sections of pyrimidine enrichment where no association occurs.

In a second situation, it may be desirable to target purine-rich regions along a single strand of a duplex but to provide bonding using different motifs. For example, the GT motif may offer some advantages when G/C-rich regions are encountered because of the pH dependence of binding of the cytosine residues in the oligomer. Thus, homopurine regions rich in guanidine may advantageously be coupled using the GT motif. On the other hand, it may be desirable to access adenine-rich regions using the CT motif. A change in motif of binding along a single strand of the duplex must be coupled with an inversion of polarity in the oligomer to correspond in the change in motif.

In the third and fourth situations, it may be desired to cross over and switch back and forth between purine-rich regions on opposite strands of the duplex. This can be done, for a single crossover, by either inverting the polarity of the targeting oligonucleotide

using a constant motif or by maintaining the polarity constant and changing the binding motif.

Thus, in some instances, the ability of oligonucleotide sequences to hybridize to double-stranded duplex DNA is enhanced by providing oligonucleotides with inverted polarity either so that the binding oligonucleotide can skip from one complementary strand in the duplex to the other as its polarity shifts, or so that advantage can be taken of the alternate motif. In its simplest embodiment, there is a single inversion of polarity in the binding oligonucleotide; of course, inversions can be inserted in a number depending on the DNA duplex target sequence.

Thus, in one aspect, the invention is directed to oligonucleotide sequences containing at least two tandem sequences of opposite polarities and thus at least one linkage which inverts the polarity of the oligonucleotide, and to methods of preparing and using these oligonucleotides. The inversion of polarity may, if desired, be combined with an alternation in the binding motifs with regard to triple helix formation.

The invention also comprises methods for binding an oligonucleotide to tandem portions of both strands of a double-helical polynucleotide duplex comprising the step of coupling the target double-helical polynucleotide duplex with an oligonucleotide to form a triplex, wherein the oligonucleotide is characterized by a first sequence of nucleotides capable to bind a portion of the first strand of the duplex, followed by a second sequence of nucleotides capable to bind a portion on the second strand of the duplex which is proximal to said target portion on the first strand wherein the second sequence has either opposite polarity or an alternate binding motif to the first sequence.

In still another aspect, the invention relates to oligonucleotides characterized by alternating regions rich in thymine residues followed by regions rich in guanine residues. These oligonucleotides have alternating motifs for association with DNA duplexes; another invention aspect relates to methods for binding duplexes using these, as stated above.

The invention also relates to a method to bind at least two proximal portions of a single strand of a duplex using oligonucleotides with inverted polarity.

Brief Description of the Drawings

Figure 1 shows a 1,4-dihydroxylmethyl benzene-mediated linkage between two adjacent ribosyl residues and two adjacent xylosyl residues.

Figure 2 shows an outline of a reaction scheme to include a "switchback" of the type illustrated in Figure 1.

Figure 3 shows an outline of the reaction scheme to include a dimer synthon in the oligomer to obtain a "switchback" or polarity reversal.

Figure 4 shows a series of candidate duplexes containing purine-rich regions on a single strand of the duplex.

Figure 5 shows an outline of the types of oligomers envisioned.

Modes of Carrying Out the Invention

As used herein "oligonucleotide" is generic to polydeoxyribonucleotides (containing 2'-deoxy-D-ribose or modified forms thereof), i.e., DNA, to polyribonucleotides (containing D-ribose or modified forms thereof), i.e., RNA, and to any other type of polynucleotide which is an N-glycoside or C-glycoside of a purine or pyrimidine base, or modified purine or pyrimidine base.

The term "nucleoside" or "nucleotide" will similarly be generic to ribonucleosides or ribonucleotides, deoxyribonucleosides or deoxyribonucleotides, or to any other nucleoside which is an N-glycoside or C-glycoside of a purine or pyrimidine base, or modified purine or pyrimidine base. Thus, the stereochemistry of the sugar carbons may be other than that of D-ribose in certain limited residues, as further described below.

"Nucleoside" and "nucleotide" include those moieties which contain not only the known purine and pyrimidine bases, but also heterocyclic bases which have been modified. Such modifications include alkylated purines or pyrimidines, acylated purines or pyrimidines, or other heterocycles. Such "analogous purines" and "analogous pyrimidines" are those generally known in the art, many of which are used as chemotherapeutic agents. An exemplary but not exhaustive list includes 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-fluorouracil, 5-bromouracil, 5-carboxymethylamino-methyl-2-thiouracil, 5-carboxymethylaminomethyl uracil, dihydrouracil, inosine, N6-isopentenyl-adenine, 1-methyl-adenine, 1-methylpseudouracil, 1-methylguanine, 1-methyl-inosine, 2,2-dimethylguanine, 2-methyladenine, 2-methyl-guanine, 3-methylcytosine, 5-methylcytosine, N6-methyl-adenine, 7-methylguanine, 5-methylaminomethyl uracil, 5-methoxy aminomethyl-2-thiouracil, beta-D-mannosyl-queosine, 5'methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, wybutoxosine, pseudouracil, queosine, 2-thio cytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, and 2,6-diaminopurine. "Nucleosides" or "nucleotides" also include those which contain modifications in the sugar

moiety, for example, wherein one or more of the hydroxyl groups are replaced with halogen, aliphatic groups, or functionalized as ethers, amines, and the like. Examples of modified nucleosides or nucleotides include, but are not limited to:

2-aminoadenosine	2'-deoxy-2-aminoadenosine
5-bromouridine	2'-deoxy-5-bromouridine
5-chlorouridine	2'-deoxy-5-chlorouridine
5-fluorouridine	2'-deoxy-5-fluorouridine
5-iodouridine	2'-deoxy-5-iodouridine
5-methyluridine	(2'-deoxy-5-methyluridine is the same as thymidine)
inosine	2'-deoxy-inosine
xanthosine	2-deoxy-xanthosine

Furthermore, as the α anomer binds to duplexes in a manner similar to that for the ß anomers, one or more nucleotides may contain this linkage or a domain thereof. (Praseuth, D., et al., Proc Natl Acad Sci (USA) (1988) 85:1349-1353).

The switchback oligonucleotides of the present invention may be of any length, but lengths of greater than or equal to about 10 nucleotides, and preferably greater than about 15, are preferred. However, the longer oligonucleotides may also be made, particularly those of greater than 50 nucleotides or greater than 100 nucleotides. Oligonucleotides may contain conventional internucleotide phosphodiester linkages or may contain modified forms such as phosphoramidate linkages. These alternative linking groups include, but are not limited to embodiments wherein a moiety of the formula P(O)S, $P(O)NR_2$, P(O)R, P(O)OR', CO, or $CONR_2$, wherein R is H (or a salt) or alkyl (1-12C) and R' is alkyl (1-6C) is joined

to adjacent nucleotides through -O- or -S-. Not all such linkages in the same oligomer need to be identical.

Inversions of polarity can also occur in "derivatives" of oligonucleotides. "Derivatives" of the oligomers include those conventionally recognized in the art. For instance, the oligonucleotides may be covalently linked to various moieties such as intercalators, substances which interact specifically with the minor groove of the DNA double helix and other arbitrarily chosen conjugates, such as labels (radioactive, fluorescent, enzyme, etc.). These additional moieties may be derivatized through any convenient linkage. For example, intercalators, such as acridine can be linked through any available -OH or -SH, e.g., at the terminal 5' position of RNA or DNA, the 2' positions of RNA, or an OH, NH_2, COOH or SH engineered into the 5 position of pyrimidines, e.g., instead of the 5 methyl of cytosine, a derivatized form which contains, for example, $-CH_2CH_2NH_2$, $-CH_2CH_2CH_2OH$ or $-CH_2CH_2CH_2SH$ in the 5 position. A wide variety of substituents can be attached, including those bound through conventional linkages. The indicated -OH moieties in the oligomers may be replaced by phosphonate groups, protected by standard protecting groups, or activated to prepare additional linkages to other nucleotides, or may be bound to the conjugated substituent. The 5' terminal OH may be phosphorylated; the 2'-OH or OH substituents at the 3' terminus may also be phosphorylated. The hydroxyls may also be derivatized to standard protecting groups.

Oligonucleotides or the segments thereof of 5'→3' or 3'→5' polarity are conventionally synthesized. Methods for such synthesis are found, for example, in Froehler, B., et al., Nucleic Acids Research (1986) 14:5399-5467; Nucleic Acids Research (1988) 16:4831-

4839; Nucleosides and Nucleotides (1987) 6:287-291; Froehler, B., Tet Lett (1986) 27:5575-5578.

In general, there are two commonly used solid phase-based approaches to the synthesis of oligonucleotides containing conventional 3'→5' or 5'→3' linkages, one involving intermediate phosphoramidites and the other involving intermediate phosphonate linkages. In the phosphoramidite based synthesis, a suitably protected nucleotide having a cyanoethylphosphoramidite at the position to be coupled is reacted with the free hydroxyl of a growing nucleotide chain derivatized to a solid support. The reaction yields a cyanoethyl-phosphite, which linkage must be oxidized to the cyanoethylphosphate at each intermediate step, since the reduced form is unstable to acid. The H-phosphonate-based synthesis is conducted by the reaction of a suitably protected nucleoside containing an H-phosphonate moiety at a position to be coupled with a solid phase-derivatized nucleotide chain having a free hydroxyl group, in the presence of a suitable activator to obtain an H-phosphonate diester linkage, which is stable to acid. Thus, the oxidation to the phosphate or thiophosphate can be conducted at any point during the synthesis of the oligonucleotide or after synthesis of the oligonucleotide is complete. The H-phosphonates can also be converted to phosphoramidate derivatives by reaction with a primary or secondary amine in the presence of carbon tetrachloride. To indicate the two approaches generically, the incoming nucleoside is regarded as having an "activated phosphite/phosphate" group.

Variations in the type of internucleotide linkage are achieved by, for example, using the methyl phosphonate precursors rather than the H-phosphonates per se, using thiol derivatives of the nucleoside moieties

and generally by methods known in the art. Nonphosphorous based linkages may also be used, such as the formacetal type linkages described and claimed in copending applications U.S. Serial Nos. 426,626 and 448,914, filed on 24 October 1989 and 11 December 1989, both assigned to the same assignee and both incorporated herein by reference.

Thus, to obtain an oligonucleotide segment which has a 3'→5' polarity, a nucleotide protected at the 5' position and containing an activated phosphite/phosphate group at the 3' position is reacted with the hydroxyl at the 5' position of a nucleoside coupled to a solid support through its 3'-hydroxyl. The resulting condensed oligomer is deprotected and the reaction repeated with an additional 5'-protected, 3-'phosphite/phosphate activated nucleotide. Conversely, to obtain an oligomeric segment of 5'→3' polarity, a nucleotide protected in the 3' position and containing an activated phosphite/phosphate in the 5' position is reacted with a nucleotide oligomer or nucleoside attached to a solid support through the 5' position, leaving the 3'-hydroxyl available to react. Similarly, after condensation of the incoming nucleotide, the 3' group is deprotected and reacted with an additional 3'-protected, 5'-activated nucleotide. The sequence is continued until the desired number of nucleotides have been added.

In addition to employing these very convenient and now most commonly used, solid phase synthesis techniques, oligonucleotides may also be synthesized using solution phase methods such as triester synthesis. These methods are workable, but in general, less efficient for oligonucleotides of any substantial length.

The oligonucleotides of the invention which are designed to target duplexes for triplex formation may have, as stated above, either uniform or mixed motifs and

may or may not contain regions of inverted polarity. With respect to variation in motif, portions of the oligomers may contain regions designed to effect CT-type binding; additional regions in the same oligomer are designed to effect GT-type binding. The regions designed to effect CT-type binding will be enriched in pyrimidine residues, as in this mode, the T-residues can be considered to target A-residues and C-residues can be considered to target G-residues in the duplex and the targeted purine regions are read in an antiparallel orientation with respect to the oligonucleotide. A region of the oligonucleotide of the invention which effects CT binding will be pyrimidine rich and will contain a sequence designed to read a purine-enriched sequence on a strand of a target duplex in a parallel orientation.

Where all of the oligonucleotide is designed to effect the Hogan mode, of course, the binding sequences will be enriched in purines. Thus, for mixed motif oligonucleotides, regions of pyrimidine enrichment will alternate with purine enrichment regions. Where the alteration in motif is combined with inverted polarity, in general, the transition from pyrimidine enrichment to, for example, purine enrichment will generally coincide with the region of inversion of polarity.

Whatever the design of the oligonucleotide, the strand of the duplex containing a purine-enriched region is formally targeted in order to rationalize the rules. It is understood that this "targeting" of the purine-rich region is a matter of formality and the rules could be rewritten in terms of the pyrimidine-enriched strand, if desired.

In their most general form, the inverted polarity oligonucleotides of the invention contain at least one segment along their length of the formula:

3'-----5'--C--5'-----3' (1)

or

5'-----3'--C--3'-----5' (2)

where -C- symbolizes any method of coupling the nucleotide sequences of opposite polarity.

In these formulas, the symbol 3'----5' indicates a stretch of oligomer in which the linkages are consistently formed between the 5' hydroxyl of the ribosyl residue of the nucleotide to the left with the 3' hydroxyl of the ribosyl residue of the nucleotide to the right, thus leaving the 5' hydroxyl of the rightmost nucleotide ribosyl residue free for additional conjugation. Analogously, 5'----3' indicates a stretch of oligomer in the opposite orientation wherein the linkages are formed between the 3' hydroxyl of the ribosyl residue of the left nucleotide and the 5' hydroxyl of the ribosyl residue of the nucleotide on the right, thus leaving the 3' hydroxyl of the rightmost nucleotide ribosyl residue free for additional conjugation.

The linkage, symbolized by -C-, may be formed so as to link the 5' hydroxyls of the adjacent ribosyl residues in formula (1) or the 3' hydroxyls of the adjacent ribosyl residues in formula (2), or the "-C-" linkage may conjugate other portions of the adjacent nucleotides so as to link the inverted polarity strands. "-C-" may represent a linker moiety, or simply a covalent bond.

It should be noted that if the linkage between strands of inverted polarity involves a sugar residue,

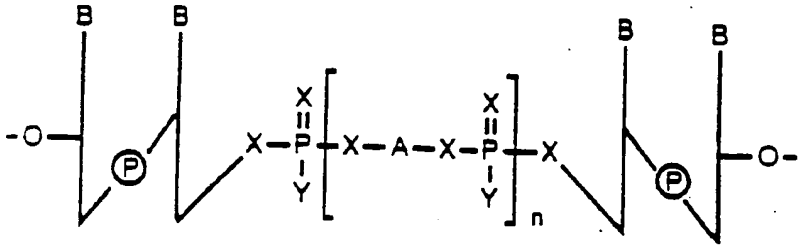
either the 3' or 2' position can be involved in the linkage, and either of these positions may be in either R or S configuration. The choice of configuration will in part determine the geometry of the oligomer in the vicinity of the linkage. Thus, for example, if adjacent 3' positions are used to effect a covalent linkage, less severe deformation of the oligonucleotide chain will generally occur if both 3' hydroxyls involved in the linkage are in the conventional R configuration. If they are both in the S configuration, this will result in a "kink" in the chain.

In addition to the use of standard oligonucleotide synthesis techniques or other couplings to effect the 5'-5' or 3'-3' linkage between ribosyl moieties, alternative approaches to joining the two strands of inverted polarity may be employed. For example, the two appended bases of the opposing termini of the inverted polarity oligonucleotide sequences can be linked directly or through a linker, or the base of one can be linked to the sugar moiety of the other. Any suitable method of effecting the linkage may be employed. The characterizing aspect of the switchback oligonucleotides of the invention is that they comprise tandem regions of inverted polarity, so that a region of 3'→5' polarity is followed by one of 5'→3' polarity, or vice versa, or both.

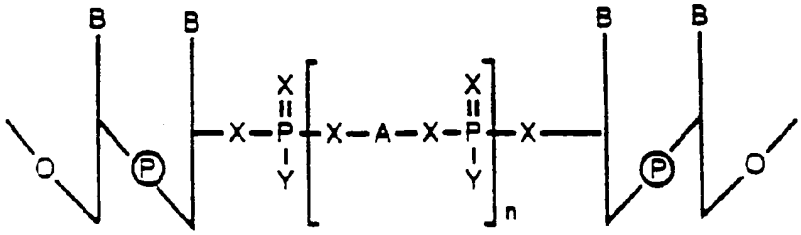
Depending on the manner of coupling the segments with inverted polarity, this coupling may be effected by insertion of a dimeric nucleotide wherein the appropriate 3' positions of each member of the dimer or the 5' positions of each member of the dimer are activated for inclusion of the dimer in the growing chain, or the conventional synthesis can be continued but using for the condensing nucleotide a nucleotide which is protected/activated in the inverse manner to that which

would be employed if the polarity of the chain were to remain the same. This additional nucleotide may also contain a linker moiety which may be included before or after condensation to extend the chain.

For example, in one illustrative embodiment of the formulas (1) and (2), these compounds include inversion-conferring linkages of the formulas:

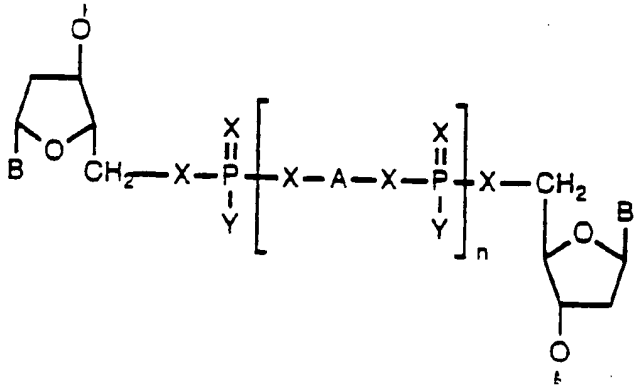


(1')

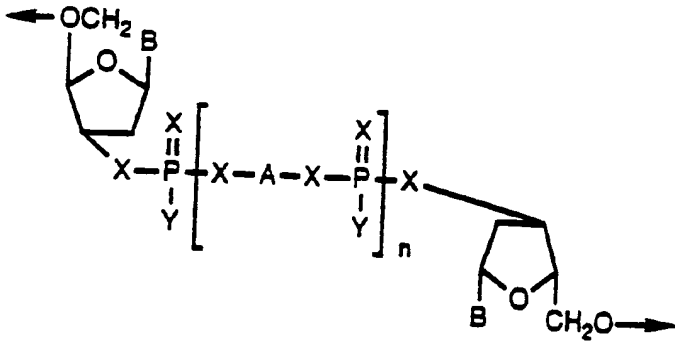


(2')

or the more complete representation:



(1')



(2')

wherein:

B is any purine or pyrimidine base, modified purine or pyrimidine base, or any desired corresponding moiety of an analogous nucleotide and (P) represents a phosphodiester linkage or a conventional substitute therefor, such as methyl phosphonate;

Y is H, -OR, -SR, $-NR_2$, O^-, or S^-;

X is O, S, or NR;

wherein each R is independently H, alkyl (1-12C), aryl(6-12C), aralkyl(7-20C) or alkaryl(7-20C);

n is 0 or 1; and

A is the residue of a linker group.

Thus, in these representations, the bold line refers to the sugar, whereas B represents the bases. The (P) within the diagonal line in the diagram denotes a phosphodiester or analogous bond. This diagonal line joins the end of one bold line and the middle of another. These junctions refer to the 5'-OH and 3'-OH, respectively.

This type of linkage is convenient because -C- can be incorporated sequentially using the standard solid phase synthesis techniques. Although shown specifically to effect a 5'-5' or 3'-3' linkage, the linking portion per se can be used to couple sugar-sugar, sugar-base or base-base on adjacent switchback nucleotide residues.

Also, any linkage form can be included using a prelinked dimer in the solid phase sequence.

When n is 0 in the above embodiment, the 3'-3' or 5'-5' linkage is simply formed using standard oligonucleotide synthesis techniques wherein the nucleotide to be added to the sequence is protected and activated in the opposite orientation from that which would be used if the original chain polarity were followed. When n=1, a linker is utilized to effect the inverted polarity linkage. There is no theoretical reason that n cannot be >1; however, generally it is more convenient to limit the synthesis to the intermediation of one linker.

When a linker moiety is employed, the phosphite/phosphate activated linker can be included directly in the continuing oligonucleotide synthesis, followed by coupling to the first nucleotide of the inverted sequence or the first such nucleotide can be supplied already derivatized to the phosphite/ phosphate activated linker. In general, the linker comprises a diol or diamine, the residue of which appears as "A" in formulas 1' and 2'. Thus, in a typical synthesis protocol, one hydroxyl (or amino) of the diol (or diamine) is protected and the other is an activated phosphite/phosphate. This protected form can be coupled to the oligonucleotide chain attached to the solid support and then deprotected and reacted with the subsequent nucleotide residue.

Similar diol or diamine type (or disulfhydryl or hydroxyl/sulfhydryl type) linkers are also convenient when the linkage between inverted polarity segments is to be effected between adjacent bases or between a base and a sugar moiety, or these can be used to link adjacent sugars directly without the inclusion of the phosphodiester or analog thereof. In these instances, it

is generally more convenient to synthesize the switchback nucleotide dimer independently, and then to insert the dimer, again using standard oligonucleotide synthesis techniques, into the oligonucleotide to be formed. Alternate linker functionalities can be convenient when adjacent base moieties are to be used, however, in general, convenient forms of linkers are those derived from dihydroxy, diamino (or disulfhydryl or hydroxyl/sulfhydryl) compounds which can be suitably protected and activated so as to integrate them into the standard oligonucleotide synthesis protocol or otherwise used to obtain inverted dimeric nucleotides.

The significant step in the integration of these linkers, however, is that the subsequent additions to the oligomer, after the linker is inserted, are activated and deprotected nucleotides having opposite polarity from that of the preceding portion of the sequence.

Thus, illustrative suitable linkers are or include residues of diols of the following formulas or their analogous diamines (or alcohol amines). For ease of representation, the diol structures are used, but it should be kept in mind that either or both hydroxyl functionality may be replaced by an amino group or a sulfhydryl group.

$HO(CH_2)_{n1}OH$, wherein n1 is an integer that is usually 1-15, but can also be in an extended form. One or more of the $-CH_2-$ groups may be replaced by O, S or NH, provided such replacement is not adjacent to a heteroatom. (When integrated into formula 1' or 2', therefore, this linker will give "A" as a residue of the formula $-(CH_2)_{n1}-$).

In particular, the diol may represent a polyethylene glycol of the formula $HO(CH_2CH_2O)_{n2}H$, wherein n2 is an integer of 1-5.

The linker may also contain unsaturation, so that it may be of the exemplary formulas:

$HOCH_2(CX_2CX_2)_{n3}CH_2OH$, wherein n3 is an integer of 1-7 and each pair of X or adjacent C is independently H or a π bond; or

$HOCH_2(CX_2CX_2)_{n4}CH_2(CX_2CX_2)_{n5}CH_2OH$, wherein n4 and n5 are integers of 0-7 and wherein the sum of n4 and n5 is not greater than 7 and wherein each pair of X or an adjacent C is independently H or together are a > bond.

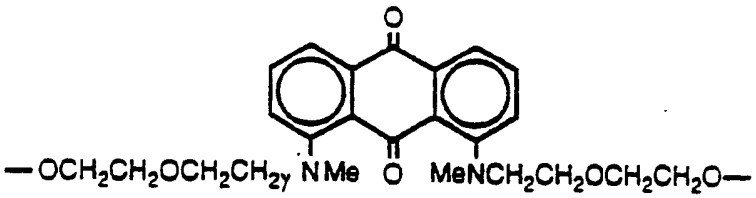
In these embodiments also, one or more methylene groups may be replaced, provided it is not adjacent to an additional heteroatom, by O, S or NH.

The dihydroxy, diamino or equivalent linker compound may also be cyclic, either non aromatic or aromatic. Nonaromatic embodiments include diols such as cis- or trans-3-4-dihydroxyfuran, cis- or trans-2-hydroxymethyl-3-hydroxyfuran, and cis- or trans-2-hydroxymethyl-4-hydroxyfuran, said furan either further unsubstituted, or further substituted with one or two noninterfering alkyl(1-4C) substituents, or may include N-heterocycles such as piperazine or piperidine.

Linkers containing aromatic rings may include residues of 1,2-dihydroxymethylbenzene; 1,4 dihydroxy methylbenzene; 1,3-dihydroxymethyl benzene; 2,6-dihydroxy methylnaphthalene; 1,5-dihydroxymethylnaphthalene; 1,4-bis(3-hydroxy propenyl)benzene; 1,3-bis(3-hydroxy propenyl)benzene; 1,2-bis(3-hydroxypropenyl) benzene; 2,6-bis(3-hydroxypropenyl)naphthalene; 1,5-bis(3-hydroxypropenyl)naphthalene, 1,4-bis(3-hydroxypropynyl)benzene; 1,3-bis(3-hydroxypropynyl)benzene; 1,2-bis(3-hydroxypropynyl) benzene; 2,6-bis(3-hydroxypropynyl)naphthalene; and 1,5-bis(3-hydroxypropynyl)naphthalene. Figure 1 shows the coupling using 1,4-dihydroxymethylbenzene as it bridges either two ribosyl or two xylosyl residues. As a portion of the inverted

polarity oligonucleotides, the DMT and phosphonate residues shown would be replaced by extended nucleotide strands. Similar representations could include others of the above linkers, in particular, 1,2- and 1,3-derivatized dihydroxymethyl benzene.

In addition, the linker may carry additional functional groups, such as anthraquinone and be fairly complex; an example of this type of linker is:



The length and type of internucleotide linkage at the inverted junction will depend in part on the charge concentration (e.g., polyphosphodiester groups may be too highly concentrated in charge) and on the distance required to span the major groove in the duplex in order to achieve the required triple helix binding. It is presently considered that spanning the two strands of the duplex through a 5'-5' switchback involves no null bases, while a 3'-3' switchback involves 1-4 null bases in the duplex target. In general, the oligomer spacing accounts for 0-4 null bases, depending on the embodiment. (A "null" base refers to a base pair in the DNA duplex that does not hydrogen bond to the third strand moiety.) The length of the linker can be adjusted accordingly. The proper length and type of linkage may be determined by those of ordinary skill in the art using routine optimization procedures.

A particular application of a switchback or inverted polarity motif lies in stabilization of oligonucleotides with respect to nuclease degradation. Even a single inversion of nucleotide linkage, preferably at the 3' terminus, will result in enhanced stability. As illustrated in Example 6 below, oligonucleotides with an inverted base moiety at the 3' terminus, either through a phosphodiester or derivatized phosphoramidate linkage exhibit enhanced stability in the presence of fetal calf serum.

Synthesis Methods

For the embodiments of formulas 1' and 2', the synthesis of oligonucleotides having inverted polarity may be accomplished utilizing standard solid phase synthesis methods.

This oligonucleotide chain elongation will proceed in conformance with a predetermined sequence in a series of condensations, each one of which results in the addition of another nucleotide. Prior to the addition of a nucleoside having an activated phosphite/ phosphate, the protecting group on the solid support-bound nucleotide is removed. Typically, for example, removal of the commonly-employed dimethoxytrityl (DMT) group is done by treatment with 2.5% v/v dichloroacetic acid/dichloromethane, although 1% w/v trichloroacetic acid/dichloromethane or $ZnBr_2$-saturated nitromethane, are also useful. Other deprotection procedures suitable for other protecting groups will be apparent to those of ordinary skill in the art. The deprotected nucleoside or oligonucleotide bound to solid support is then reacted with the suitably protected nucleotide containing an activated phosphite/ phosphate. After each cycle the carrier bound nucleotide is preferably washed with anhydrous pyridine/ acetonitrile (1:1, v/v), again

deprotected, and the condensation reaction is completed in as many cycles as are required to form the desired number of congruent polarity internucleotide bonds which will be converted to phosphoramidates, phosphorodithioates, phosphorothioates or phosphodiesters as desired.

In one embodiment, to provide the switchback, the incoming activated, protected nucleoside is provided in the opposite polarity to the support-bound oligomers. Thus, for example, where the support-bound oligomer is 3'→5', the deprotected 5' hydroxyl is reacted with a 3'-protected, 5'-activated monomer, and the synthesis continued with monomers activated at the 5' position and protected at the 3' position.

In another embodiment, to provide a linker in the switchback, a molecule having one end which is activated for condensation (such as a hydrogen phosphonate) to the support-bound oligonucleotide and another end which is a protected hydroxyl group (or protected thio group) is condensed onto the support-bound oligonucleotide. The linker group is condensed and deprotected using the same conditions as those used to condense and deprotect the protected nucleoside hydrogen phosphonate. Subsequent extension of the oligonucleotide chain then uses oligonucleotide residues which are activated and protected in the opposite manner from those used to synthesize the previous portion of the chain.

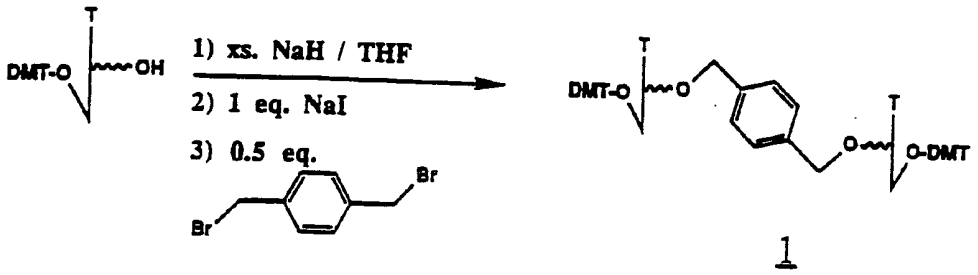
One approach to this synthesis, using a linker already derivatized to two nucleotide residues which will be included in each portion of the strand is illustrated in Figure 2. The 5'→3' nucleotide portion of the strand is coupled, as conventionally, to solid support. The linker is derivatized to two nucleotide residues through their 3' positions; the remaining 5' positions are derivatized by the protecting group DMT in one nucleotide

residue and a phosphonate residue in the other. The derivatized linker is coupled to the solid supported strand under standard reagent conditions and then deprotected conventionally. Further standard nucleotide coupling results in extension of the chain in the 3'→5' orientation.

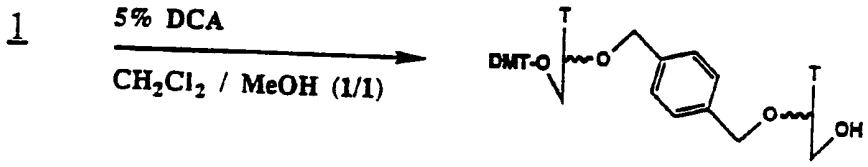
The reactions to illustrate the formation of the 3'-3' coupled synthon used in Figure 2, wherein is exemplified linkage of the 3' positions in adjacent sugar residues through 1,4-dihydroxymethylbenzene (dibromomethylbenzene) is shown in Reaction Scheme 1.

In general, the first step shown is conducted at about 45°C for 24 hours and yields about 80-95% yield of the doubly-protected synthon shown as Formula 1, which is then partially purified through flash chromatography. The second step which constitutes partial deprotection to obtain the monoprotected form of the compound, shown as Formula 2, is conducted at room temperature and takes only about 15 minutes. A mixture of products results, and the crude mixture can be used in the third step of the reaction which yields the phosphonate derivative usable in synthesis. As shown in Figure 2, a 5'→3' nucleotide chain derivatized as solid support is initially reacted with the synthon, followed by deprotection and subsequent reaction with 5' activated 3' protected nucleosides.

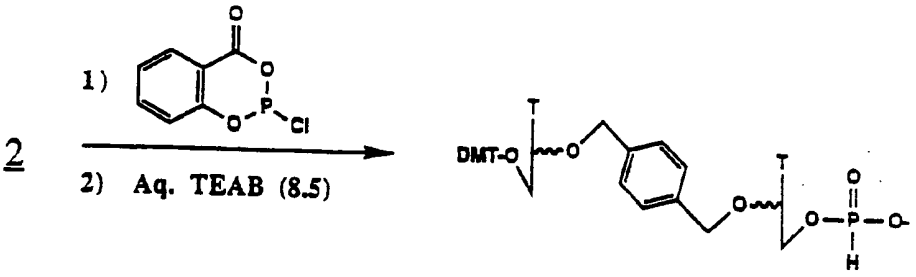
Reaction Scheme 1



1



2



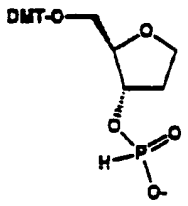
3

If coupling of the inverted portion of the oligonucleotide is through an internucleotide linkage conjugating the bases of adjacent nucleotides or the base of one nucleotide to the ribosyl moiety of the other, or adjacent ribosyl residues through linkages which do not involve activated phosphite/phosphate, it is preferable to form the dimeric nucleotide, which is then included in the synthesis in suitably activated and protected form. For example, adjacent methyl cytosines or thymidines may be linked through the methyl groups at the 5-positions of the pyrimidine rings using a variety of techniques by converting the 5-position to, for example, hydroxymethyl, allyl amine, acrylic acid, or propenyl residues, as is commonly practiced. These reactive groups can then be further coupled through bifunctional linkers or by suitable alternate condensation to obtain dimeric forms of the methyl cytidine or thymidine, or mixed nucleosides. For inclusion of the dimer in the oligonucleotide of inverted polarity, the dimer is protected, if needed, in, for example, both 5' positions and activated in one 3' position and protected in the other for continuation of the synthesis. Extension of the chain continues from the included dimer using nucleosides of inverted protection/activation patterns.

In one example, for a dimer wherein adjacent 5-positions of the bases are linked through $-(CH=CH-CH_2NH)_2CO$, the inclusion of this dimer to obtain a 5'-5' link can be shown diagrammatically in Figure 3 where (S) = polymeric or other solid phase support, Pr_B = a DMT protecting group; Pr_C is a trimethyl acetyl protecting group; P_A = activated phosphite/phosphate; and (P) is as defined above.

Dimers may also be formed between adjacent sugars, and the resulting dimers used as above in standard synthesis. For example, the 3' positions of two

ribosyl, xylosyl or ribosyl/xylosyl moieties on adjacent nucleotides may be linked through a p-dihydroxymethyl benzene or other linker such as 1,3-propylene glycol or ddR of the formula:



and the 5' positions of the dimer used in subsequent synthesis. (For these latter two linkers, standard incorporation into the oligomer synthesis scheme can also be used.) In this case one 5'-position is protected with a DMT and the other is activated phosphite/phosphate. Conversely, for adjacent sugars linked through the 5' position, one 3'-position is protected and the other activated. As stated above, the geometry of the oligonucleotide at the linkage site will be affected by the chirality of the 3' carbons involved in the linkage.

As stated above, all of the internucleotide linkages in the resulting oligomer need not be identical. By use of appropriate synthesis techniques, some can be phosphodiesters, some phosphonates, some phosphoramidates, etc.

As set forth above, the inverted polarity oligonucleotides of this invention may be derivatized. One convenient method to form such derivatization is through the phosphoramidate linkage. The amine which is utilized to form the phosphoramidate may employ substituents that can confer useful properties to the oligonucleotide. For example, if an amine linked to a

polyethylene glycol, a polypeptide or a lipophilic group is utilized, such a group may facilitate transport of the oligonucleotide through the cell membranes, thus increasing the cellular uptake of the oligonucleotide. A substituent on the amine may also include a group which affects the target DNA to which the oligonucleotide will bind such as providing covalent linkages to the target strand to facilitate cleavage or intercalation of the switchback oligonucleotide to the target strand. The substituents on the amine may contain chromophoric groups such as fluorescein or other labels, including radioactive labels, chelating agents and metal chelated ions, to label the oligonucleotide for identification. The substituents may thus also serve a cutting function (i.e., a site for cutting the duplex) or a receptor function such as a receptor ligand. The substituents on the amine which form the phosphoramidate linkage may thus be virtually any moiety which does not prevent the oligonucleotide from binding to the target duplex.

More than one derivatizing moiety may also be used as two or more phosphoramidate linkages need not contain the same substituents. This may be accomplished by generating a first nucleotide hydrogen phosphonate linkage and then oxidizing it with a first amine, generating a second hydrogen phosphonate linkage and oxidizing it with a second (different) amine.

While the formation of the phosphoramidate linkage provides a convenient method for attaching the groups which derivatize the oligonucleotide to confer useful properties, other methods may also be used. The useful substituents may be attached to the sugar moieties or to the bases, or by any other method generally known in the art.

After completion of the synthesis, the oligonucleotide is separated from the carrier using

conventional methods, such as, by incubation with concentrated ammonium hydroxide. Any protecting groups (for example, on the purine or pyrimidine bases) may be removed using, for example, the conventional concentrated ammonia reagent. The oligonucleotide is then purified by conventional means such as HPLC, PAGE (polyacrylamide gel electrophoresis) or any other conventional technique.

It will be understood that while the above method has been described in connection with use of a solid state carrier, it is also possible to conduct the synthesis without the use of a solid state support. In such an event, in place of the support a 3'-hydroxy protecting group which is different from the 5' protecting group used in the course of the condensation, may be utilized so that the 5' protecting group may be selectively removed while the 3' protecting group remains intact.

Binding Properties

The oligonucleotides of the invention including those with inverted polarity are designed to effect triplex formation with target oligonucleotide duplexes. In vitro conditions for the triplex formation are variable, but in order to be maximally effective in use for the treatment of disease or for analysis as described hereinbelow, it is generally preferred that the triplex formation be effected under physiological conditions. The manner of triplex formation will depend, of course, on the design of the oligonucleotide.

It will be understood that those oligomers which are designed to participate in CT-type motifs will contain, in those regions intended for CT binding, mostly pyrimidine-based nucleotides. Those regions of the oligonucleotides intended to participate in GT-type binding will contain mainly purine-type nucleotides. For

oligonucleotides of the invention which maintain the same binding motif but are intended to cross over between the strands of the target duplex, an inversion of polarity will be provided. Thus, the nucleotides will comprise, on one side of the 3'-3' (or 5'-5') inversion, bases which bind to one strand of the duplex according to the motif chosen, with the bases on the other side of the 3'-3' (or 5'-5') junction selected to be bases which will bind to the subsequent bases on the opposite strand of the duplex according to the same motif.

In this manner triple helix recognition may be extended by switching recognition from one strand of the duplex to the other and then back again, if desired. Also, certain nucleases may be blocked, since the oligonucleotides according to the present invention can present ends not recognizable by exonucleases. Thus, oligonucleotides having two 5'-ends, will be resistant to 3'-exonucleases.

Since the switchback oligonucleotides of the invention are intended to expand the strength of binding to duplex DNA, the sequence of nucleotides in each portion of the oligonucleotide is determined by the sequence of bases in the target duplex. For the CT motif, target duplex sequences which contain multiple adenyl residues in a homopurine region of one chain, followed by a region of homopurines comprising guanines in the opposite strand will mandate a switchback oligonucleotide which is polyT in the polarity opposite to the polyA tract followed by polyC in the polarity opposite to that of the polyG tract. Alternating A/G sequences in the first strand of the target duplex will mandate alternating T/C sequences in a first region of oligomer parallel to the enriched purine target, followed by a sequence of inverted polarity which is parallel to the second strand sequential sequence in the duplex.

It will be understood, of course, the GT motif may be maintained throughout an inverted polarity oligomer designed to cross over strands in a duplex which are enriched in guanine residues such as the sequence 5'-GGGGCCCC-3' and its complement. This oligomer would be 3'-GGGG-GGGG-3'.

In an alternate approach to providing an oligonucleotide which will cross over between the two strands of the duplex, the oligonucleotide will be provided in a single polarity, but with proximal regions of differing binding motifs. Thus, for example, if the target oligonucleotide has a region of multiple adenyl residues in a homopurine region of one chain, followed by a region of homopurines comprising guanine in the opposite strand, as described above, an alternate approach would be to provide an oligonucleotide containing polyT region of polarity parallel to that of the polyA tract, followed by polyG in the same polarity. In general, this approach is appropriate for target oligonucleotides of this type, wherein the oligomers of a single polarity will comprise regions rich in thymine proximal to regions rich in guanine.

The oligonucleotides of inverted polarity are also useful when it is desired to target only one strand of the duplex in order to effect triplex formation. Under these circumstances, the inversion of polarity will be accompanied by an alteration of the binding motif. Such oligomers are useful when the duplex contains regions of purine enrichment along a single strand. To modify the foregoing example to fit this model, a target duplex which contains on the 5'→3' strand a polyA region followed by polyG region could effectively be targeted by an oligomer which contains polyT with a 5'→3' orientation

followed by polyG with 3'→5' orientation, thus switching at the point of inverted polarity, from the CT to the GT motif.

Although, for simplicity, the oligomers of the invention have been described as fitting only one of three "categories"--i.e., inverted polarity maintaining the same motif; inverted polarity correlated with a switch in motif; single polarity and a switch in motif; it is clear that these categories can reside together on a single oligomer whose binding mode to form the triplex thus varies along the targeted duplex. Thus, an inverted polarity segment wherein the inversion is accompanied by a motif switch could be followed by an additional inversion wherein the motif is maintained. In the first inversion, accompanied by the motif switch, a single strand of the duplex will be targeted; at the second inversion wherein the motif is maintained, the oligomer will cross over to recognize the second strand of the duplex.

The sequence design of the oligomers which are intended to cross over between strands of the target duplex must take account of null bases residing in the duplexes which are effectively out of position to participate in the binding. The geometry at present is not completely understood; however, it is clear that the presence of any null bases must be accounted for. The geometry of the double helix results in a spacing requirement so that at a 3'-3' linkage in the oligonucleotide with a CT-CT motif there will be approximately 0-4, probably 1-2 essentially null bases in the duplex; there appear to be no null bases required in the case of the 5'-5' switchback. The opposite is true for combinations of 3'-3' linked GT-GT motif. The null base spacing can be provided by arbitrary nucleotide

insertions or, alternatively, the length of a linker moiety may be adjusted to compensate for this.

In addition to enhanced capability to bind to the duplex by formation of a triplex through crossing over between strands and triplex association based on target regions in a single strand, inverted polarity oligonucleotides may also form D-loops with the duplex. In this situation, the region of a first polarity may, for example, form a triplex, while the inverted portion displaces a section of one strand of the duplex to result in a substitute duplex in the relevant region. The design of the sequence of bases in the oligonucleotide takes account of this by utilization of a sequence which is designed to base pair hybridize to the target strand.

Utility and Administration

As the oligonucleotides of the invention are capable of significant duplex binding activity to form triplexes or other forms of stable association, these oligonucleotides are useful in "antisense" therapy. "Antisense" therapy as used herein is a generic term which includes the use of specific binding oligonucleotides to inactivate undesirable DNA or RNA sequences in vitro or in vivo. Because of their superior binding ability to duplex DNA, oligonucleotides of the invention are particularly helpful in this regard.

Most diseases and other conditions are characterized by the presence of undesired DNA or RNA, some of which may be in duplex form. These diseases and conditions can be treated using the principles of antisense therapy as is generally understood in the art. Antisense therapy includes targeting a specific DNA or RNA sequence through complementarity or through any other specific binding means, in the case of the present

invention by sequence-specific orientation in the major groove of the DNA double helix.

In addition to antisense applications, wherein specific sequence recognition is significant, alternate therapeutic mechanisms for oligomers of the invention can be advantageously employed. Such oligomers are generally useful as inhibitors of polymerases such as viral polymerases, to interfere with binding factors to nucleic acids such as transcription initiating or inhibiting factors, to induce the production of interferon endogenously, and so forth. The oligomers of the invention may be administered singly, or combinations of oligomers may be administered for adjacent or distant targets or for combined effects of antisense mechanisms with the foregoing general mechanisms.

In therapeutic applications, the oligomers are utilized in a manner appropriate for antisense therapy in general or to take advantage of the alternate therapeutic mechanisms set forth above. For such therapy, the oligomers can be formulated for a variety of modes of administration, including systemic, topical or localized administration. Techniques and formulations generally may be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA, latest edition. The oligomer active ingredient is generally combined with a carrier such as a diluent or excipient which may include fillers, extenders, binders, wetting agents, disintegrants, surface-active agents, or lubricants, depending on the nature of the mode of administration and dosage forms. Typical dosage forms include tablets, powders, liquid preparations including suspensions, emulsions and solutions, granules, capsules and suppositories, as well as liquid preparations for injections, including liposome preparations.

For systemic administration, injection is preferred, including intramuscular, intravenous, intraperitoneal, and subcutaneous. For injection, the oligomers of the invention are formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the oligomers may be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also included.

Systemic administration can also be by transmucosal or transdermal means, or the compounds can be administered orally. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, bile salts and fusidic acid derivatives for transmucosal administration. In addition, detergents may be used to facilitate permeation. Transmucosal administration may be through use of nasal sprays, for example, or suppositories. For oral administration, the oligomers are formulated into conventional oral administration forms such as capsules, tablets, and tonics.

For topical administration, the oligomers of the invention are formulated into ointments, salves, gels, or creams, as is generally known in the art.

In addition to use in therapy, the oligomers of the invention may be used as diagnostic reagents to detect the presence or absence of the target DNA or RNA sequences to which they specifically bind. Such diagnostic tests are conducted by hybridization through triple helix formation which is then detected by conventional means. For example, the oligomers may be labeled using radioactive, fluorescent, or chromogenic labels and the presence of label bound to solid support detected. Alternatively, the presence of a triple helix

may be detected by antibodies which specifically recognize these forms. Means for conducting assays using such oligomers as probes are generally known.

In addition to the foregoing uses, the ability of the oligomers to inhibit gene expression can be verified in in vitro systems by measuring the levels of expression in recombinant systems.

The following examples are provided to illustrate but not to limit the invention.

Example 1

Preparation of 3'-DMT-N^4-benzoyl-dC-5'-H-phosphonate

6.4g (10 mmole) of 5'-DMT N^4-benzoyl deoxy-C is dried from 100 ml of pyridine, dissolved into 100 ml of pyridine and to this is added 4g (11.8 mmole) of DMT-Cl and the reaction mixture stirred at room temperature for three days. The reaction mixture is evaporated to approximately half the volume and diluted with 100 ml of CH_2Cl_2, wash with 5% sodium bicarbonate (2 x 100 ml), dry over sodium sulfate and evaporate to dryness. The crude mixture is dissolved into 100 ml of toluene and evaporated to a foam, and this is repeated one more time. The solid is taken up in 50 ml of diethyl ether/50 ml of CH_2Cl_2 and precipitated into 900 ml of hexane at room temperature. The solid is isolated and dissolved into 15 ml of CH_2Cl_2, cool to 0°C and add 100 ml of saturated $ZnBr_2$ in isopropanol/CH_2Cl_2 (15/85) and stirred for 15 minutes. Reaction mixture is quenched into 400 ml of 1M NH_4 OAc, the organic layer separated and wash with $NaHCO_3$ (1 x 200 ml), dry over Na_2SO_4 and evaporate. Purify by silica gel chromatography (CH_2Cl_2/5% MeOH) to yield 50% of the 5'-OH product.

The 5'-OH nucleoside is dried from 50 ml of pyridine then taken up in 10 ml pyridine and 10 ml of

methylene chloride, to which is added 2 eq. of 1M PA/CH_2Cl_2 in 5 ml of pyridine. This mixture is stirred at r.t. for 15 minutes and quenched into 1M TEAB, the layers are separated, and the organic layer is washed with TEAB (1 time), dried over sodium sulfate and evaporated to dryness. The reagent PA is von Boom's Reagent, 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one. The nucleoside H-phosphonate is purified by silica gel chromatography (1% triethylamine/CH_2Cl_2) with a MeOH gradient.

Example 2

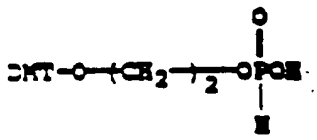
Preparation of 3'-DMT-thymidine-5'-H-phosphonate

1.2g (5 mmole) of thymidine is dried from 50 ml of pyridine, taken up in 20 ml of pyridine and under Ar is added 820 mg (5.5 mmole) of t-butyldimethyl silyl chloride in 5 ml of pyridine. The mixture is stirred at room temperature for one day, concentrated to approximately 10 ml, diluted with 75 ml of CH_2Cl_2, washed with 5% $NaHCO_3$, back extracted the aqueous with CH_2Cl_2, dried over Na_2SO_4 and evaporated. The crude nucleoside is dried from 20 ml pyridine, taken up in 30 ml pyridine and to it is added 1.7g (5 mmole) of DMT-Cl and 0.4 ml of triethylamine, after which the mixture is stirred for three days. After evaporation to approximately 10 ml, the mixture is diluted with 75 ml of CH_2Cl_2 and washed with $NaHCO_3$ (2 x 100 ml), dried over Na_2SO_4 and evaporated. The 5'-OH nucleoside is taken up into 60 ml THF and 20 ml of 1M TBAF/THF is added. After stirring for one hour, the mixture is evaporated to an oil, taken up in CH_2Cl_2 and applied to silica gel column. Yield 50%. The 5'-OH nucleoside is then converted to H-phosphonate as in Example 1.

Example 3

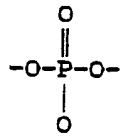
Synthesis of Oligomer Containing Switchbacks

Polynucleotide H-phosphonates condensed at the 3'-end to a solid polymer support are prepared as described by Froehler, et al., Nuc Acids Res (1988) 16:4831-4839; Nuc Acids Res (1986) 14:5399-5467; and Nucleosides and Nucleotides (1987) 6:287-291; using the DBU salt of 5'-protected nucleoside H-phosphonates. After four couplings, one coupling cycle is performed using the ethylene glycol derivative:



The polynucleotide H-phosphonate is then oxidized with aqueous I_2 (0.1M in N-methyl morpholine/water/THF, 5/5/90) to form internucleotide diester linkages. Then five coupling cycles are performed using 3'-protected nucleoside 5'-H-phosphonates, prepared as in Examples 1 and 2. After these couplings the remaining H-phosphonate linkages on the polynucleoside are oxidized with 2-methoxyethylamine in Pyr/CCl_4 (1/5/5), to generate a 10-mer with five diester linkages (one of which is with the ethylene glycol linker) and five phosphoramidate linkages (one of which is with the ethylene glycol linker). The oligomer is removed from the solid support, deprotected with concentrated NH_4OH, purified by HPLC (PRP) using an acetonitrile gradient in 50 mM aqueous TEAP. DMT is removed using 80% HOAc (R.T.) and the solvent is evaporated. The product is desalted, and isolated by evaporation.

Thus, in this manner the following are prepared, wherein P_1 represents



(having an ionization state determined by pH):

3' 5' 5' 3'
C-C-C-C-C-P_1-CH_2CH_2-P_1-C-C-C-C-C

3' 5' 5' 3'
C-T-C-T-C-P_1-CH_2CH_2-P_1-C-T-C-T-C

3' 5' 5' 3'
T-T-C-C-C-P_1-CH_2CH_2-P_1-C-C-C-T-T

3' 5' 5' 3'
T-C-T-T-T-P_1-CH_2CH_2-P_1-C-T-T-C-T

By utilizing nucleosides derivatized to solid support through the 5' portion and extending the chain with the 3'-protected, 5'-activated nucleosides of Examples 1 and 2, followed by coupling to DMT-O-CH_2CH_2-P_1 as above, followed by chain extension, with conventional 5'-protected, 3'-activated nucleosides, the following are prepared.

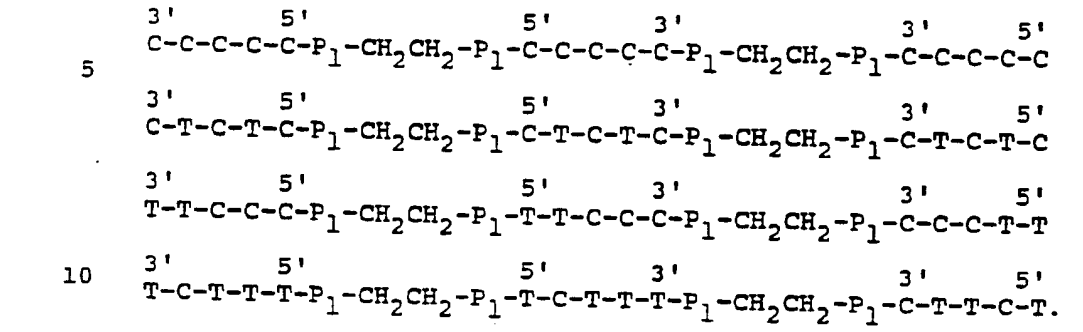
5' 3' 3' 5'
C-C-C-C-C-P_1-CH_2CH_2-P_1-C-C-C-C-C

5' 3' 3' 5'
C-T-C-T-C-P_1-CH_2CH_2-P_1-C-T-C-T-C

5' 3' 3' 5'
T-T-C-C-C-P_1-CH_2CH_2-P_1-C-C-C-T-T

5' 3' 3' 5'
T-C-T-T-T-P_1-CH_2CH_2-P_1-C-T-T-C-T

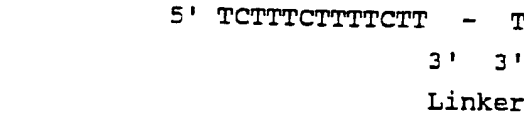
By insertion of an additional linker, the following are prepared:



Example 4

Synthesis and Assay of a Recognition Switchback

Using the methods of Example 3, a switchback 24-mer linked with propylene glycol was prepared of the formula:



The duplex DNA to which this oligomer binds has a 26-bp target region having 2 null bases to accommodate the switchback. This target region is of the formula:

5' AGAAAGAAAAGAAATTTCTTCTTTTT 3'
3' TCTTTCTTTTCTTTAAAGAAGAAAAA 5'

To assess the capacity of the switchback oligomer to bind the target region, an assay was conducted as follows. A 144-bp fragment containing four 36-bp cassettes, one of which corresponds to the desired target region, was cloned into pTZ18U, a commercially available cloning vector. The plasmid was cleaved and

labeled at both ends with 32 PdCTP, and then digested with a second restriction enzyme to eliminate the label at one end and reduce the size of target DNA. The resulting 372 bp fragment was purified using 5% acrylamide gel.

The switchback oligomer (1 mmol) was incubated with 5 x 10^4 cpm labeled target in 100 mM NaCl, 10 mM $MgCl_2$, 50 mM MES, pH 6, at room temperature for one hour.

The reaction mixtures were then treated with 0.5 mg/ml DNAse-I for 1 minute at room temperature, and extracted to terminate the digestion and remove protein. The DNA was recovered by ethanol precipitation, and the samples were loaded onto 8% polyacrylamide gel in 80% formamide and run under denaturing conditions for 2 hours at 60 watts. The gel was dried and placed on film overnight. The results showed that the oligomer provided protection against DNAse-I activity indicating formation of triple stranded structures in the target region and not in related target sequences. Protection is extended over approximately 20 bp region, as determined with a 14-mer control.

Example 5

Triplex Forming Oligomers With Respect to Target Duplexes Associated With Disease

A search through databases containing DNA sequences associated with particular disease conditions provides some illustrative targets shown in Figure 4. Figure 4A shows a region of a double-stranded portion of the Herpes simplex I genome. Several invention oligomers can be utilized to target this region.

First, an oligonucleotide of the formula:

5'-TTTTTTNTTTTTNT- - -CCCC-5' (1)
3'--3'
linker

which contains a region of inverted polarity but maintains CT motif throughout. This oligomer will effect a crossover between the upper strand wherein the thymine residues target the adenyl-rich portion of the duplex by virtue of the inverted polarity to the polyC tract which targets the polyG region in the opposite strand. (The positions designated "N" in formula (1) can be any nucleotide, as these positions are not responsible for recognition.)

An alternative approach would be to design an oligonucleotide of formula (2) which employs a motif switch from CT to GT to effect the crossover.

5'-TTTTTTNTTTTTNT - GGGG-3' (2)

Figure 4B shows a region of the human cytomegalovirus genome which encodes the 67 kd phosphorylated protein. This region invites similar options.

Figure 4C shows a portion of the gene encoding human IL-1ß. For conditions characterized by an unwanted amount of this lymphokine, triplex formation using oligomer (3) or (4) could be employed.

5'-GGGGGGG - TTTTTTTTTTT-3' (3)

3'-CCCCCCC - TTTTTTTTTT-3' (4)
5'--5'
linker

Oligomer (3) is the converse of oligomer (2); it employs a motif switch to effect a crossing over of the targeted strands. On the other hand, oligomer (4), somewhat

analogous in concept to oligomer (1), employs an inversion of polarity to effect the crossover.

Figure 4D offers an example of a region (a Varicella Zoster genome) wherein an opportunity is offered to maintain binding in at least a region of one strand. Thus, to maintain triplex formation through the GGGAAA portion of the upper strand as shown, the oligomers (5) or (6) could be employed.

5'-CCC ~ TTT-3' (5)

3'-GGG ~ TTT-3' (6)
5'--5'
linker

To effect the crossover between the A region and G region on opposite strands as shown, oligomers analogous to oligomer (1) and (2) would be needed; for binding to the region as a whole, therefore, there are a total of four possibilities: (5) in combination with (1) or (2), or (6) in combination with (1) or (2).

Figure 4E provides another example of a single strand target which is a portion of human 4EXB. A suitable oligomer has the formula:

5'-TTTTT ~ GTGTGG-5'.

Example 6

Synthesis of an Inverted N-capped Oligonucleotide

An 11-mer oligonucleotide of the sequence 5'-TTTTTCTCCAT, wherein the 3' terminal A-T linkage was modified to couple the 3' position of the adenyl residue with the 3' position of the thymidyl residue was synthesized using standard oligonucleotide synthesis methods, but with the thymidyl residue protected in the 5' position and activated in the 3' position in the last step of the oligonucleotide synthesis. Both the

phosphodiester and the methoxyethyl amine (MEA) derivative of a phosphoramidate linkage were obtained. These oligomers were 5' end labeled using gamma 32P ATP and T4 kinase. The labeled oligonucleotide was then ligated to a 10-mer (5'-TCCAGTGATT) using T4 DNA ligase in the presence of the 33-mer template: 5'-TCGCTGATGGAGAAAAAAATCACTGGAGACCTC. The internally labeled 21-mer 5'-TCCAGTGATT32pTTTTTCTCCAT was desalted with the C_8 SPE column.

The internally labeled oligonucleotides were added to serum-containing media in an H938 human T lymphoma cell culture at a concentration of 10 nM either with or without 20 μM of unlabeled oligonucleotide. The degree of degradation was determined at various time points by polyacrylamide gel analysis. Under these conditions, a control 21-mer having no inverted linkage at the 3' terminus had a half-life of approximately 3 hours; however, both the inverted diester and amidate-linked experimental 21-mers had half lives of more than 7 days.

Example 7

Synthesis of Ether-Linked Dinucleoside H-Phosphonates

The meta-xylyl linked analog of the compound of Formula 3 shown in Reaction Scheme 1 hereinabove was synthesized as described in this example. This compound contains a 5' DMT-protected thymidyl residue linked through the 3' position through a 1,3-xylyl residue and to an additional thymidine residue through the 3' position. The second thymidyl is activated at the 5' position with a phosphonate residue.

400 mg (36 mmol) of NaH (60% dispersion in mineral oil) was washed with anhydrous THF (2 x 10 mL), suspended into 20 mL anhydrous THF and to this was added 545 mg (1.0 mmol) of 5'-DMT protected nucleoside with

stirring under Ar. After evolution of H_2 had stopped 150 mg (1.0 mmol) of NaI and 132 mg (0.5 mmole) α,α'-dibromo-m-xylene were added and the reaction mixture stirred at 40°C for 24 hrs. The reaction mixture was cooled to room temperature and quenched with sat'd $NaHCO_3$, evaporated to a gum, taken-up in CH_2Cl_2 (50 mL), washed with $NaHCO_3$ (2 x 50 mL), dried over Na_2SO_4 and evaporated. The product (2 in Reaction Scheme 1) was purified by flash chromatography on silica gel using MeOH/CH_2Cl_2 gradient (0-5%) and yielded the symmetrical dimer in 95% yield. This was then dissolved into 10 mL of MeOH/CH_2Cl_2 (1/1) and to this was added with stirring 15 mL of 10% DCA in MeOH/CH_2Cl_2 (1/1), after 10 min. the reaction was quenched by the addition of sat'd $NaHCO_3$, the layers separated and the organic layer washed with sat'd $NaHCO_3$ (2 x 25 mL), dried over Na_2SO_4 and evaporated. The crude material was coevaporated from anhydrous pyridine (2 x 20 mL), taken-up in 10 mL of Pyr/CH_2Cl_2 (1/1) and added to 5 mL of a 1M solution of PA/ in Pyr/Ch_2Cl_2 (1/1) at room temperature, stirred for 15 min. and poured into 75 mL of aq. TEAB (1 M, pH=8.5). The H-phosphonate was purified by flash chromatography to yield 100 mg (25%) of the xylyl 1,3-linked analog of compound 3 in Reaction Scheme 1.

Example 8

Determination of Tm

A series of "9 x 9" oligonucleotides containing 9 nucleoside residues 5'→3', linked to 9 oligonucleotide residues linked 3'→5', were designed to form a triple helix with the target duplex of the sequence

5'-GAAAAAGAAATTTCTTCTT-3'
3'-CTTTTTCTTTAAAGAAGAA-5'

The 9 x 9 oligonucleotides were designed assuming one, two or three null bases in the duplex. Thus, the oligomer which assumes one null base pair will have the nucleotide sequence

5'-CTTTTTCTT-X-TTTCTTCTT-5'. (7)

The oligomer designed assuming two null base pairs will have the sequence

5'-CTTTTTCTT-X-TTCTTCTTT-5'. (8)

The oligomer assuming three null base pairs will have the sequence

5'-TCTTTTTCT-X-TTCTTCTTT-5'. (9)

In the above sequences, X represents a linker which is either the residue of an ortho, meta or para xylene, the residue of propylene glycol, the residue of 1,2-dideoxyribose or dT. Where the linker is a residue of ortho, meta or para xylene, the thymidyl residues shown on either side of the residue in the formulas may contain either ribose or xylose as the sugar moiety.

Thus, compounds of formulas (7), (8) and (9) were synthesized wherein X is PG, dDR or dT linked through the 3' positions of the adjacent thymidyl residues, or wherein X represents the residue of ortho, meta or para xylene and the thymidyl residues contain either ribose or xylose; in this instance, the "linker" is denoted o-xylose, m-xylose, p-xylose; o-ribose, m-ribose, p-ribose; depending on whether the sugar residues in the adjacent thymidyl residues are ribose or xylose, although the thymidyl residues are shown in the formulas.

Measurement of thermal denaturation (Tm) was conducted using a Gilford Response II temperature control spectrophotometer. The heating rate was about 0.25°C/min from 15°C-75°C. The final concentration of all three strands was about 2 μM. Buffers were prepared from 140 mM KCl/5 mM sodium phosphate/5 mM magnesium chloride and brought to pH 6.2. Prior to the Tm measurement, buffers were degassed with argon and pH adjusted to the correct value. Tm values were determined by a first derivative plot of absorbance vs. temperature.

The results are shown in Table 3.

Table 3

Tm of 9 x 9 Switchbacks (3'-3') at pH = 6.2

Linker	1 nbp	2 nbp	3nbp
o-xylose	42.1	47.6	nd
m-xylose	39.2	31.7	nd
p-xylose	37.5	33.4	nd
o-ribose	nd	34.8	26.6
m-ribose	nd	32.2	26.1
p-ribose	nd	31.4	22.3
PG	nd	33.5	26.3
ddR	nd	32.0	26.5
dT	nd	30.5	33.3

nd = not determined

These results indicate the nature of the linkage can be designed to accommodate the number of null base pairs in the target.

The binding of the oligomer to the target duplex was also assessed in a manner similar to that set forth in Example 4 as follows. About 0.1 nmol target DNA labeled with about 50,000 cpm of p32 inserted into a host vector was incubated with various concentrations of the test oligonucleotide over a range of 0.1-100 μM in 50 μl reaction buffer (20 mM MES, pH 6.0, 10 mM $MgCl_2$, 100 mM NaCl) for about 1 hour at room temperature to form the triplex. The triplex was treated with 0.2 units DNAseI for 1 min at room temperature and the reaction was stopped with 2 μl 0.5 M ETDA. A 2 μg sample of carrier tRNA was added and the reaction mixture was precipitated with ethanol. The pellet was resuspended in 3 μl 80% formamide, heated for 5 min at 90°C and run on a 6% denaturing polyacrylamide gel to obtain a footprint. Protection by the 9 x 9 mers was shown by absence of cleavage products of the target in the gel.

Example 9

Cooperative Effect of Xylose Linkers

In a manner similar to that set forth in Example 8, above, a 28-mer of the sequence

5' AGAAAAAGAAATTTCTTCTTTTTCTTTT 3'
3' TCTTTTTCTTTAAAGAAGAAAAAGAAAA 5'

was inserted into the vector for use as a target. The following oligonucleotides were designed to target this duplex assuming two null bases in the duplex.

5'-TTTCTTTTTCTTCTT-"o-xylose"-TCT-5' (10)

5'TTTTTCTTCTTT-"o-xylose"-TTCTTT-5' (11)

5'-TTTCTTTTTCTTCTT-PG-TTC-5' (12)

5'-CTTTTTCTTCTT-PG-TTCTTT-5' (13)

Formula 10 is a 15 x 3 switchback oligomer having an o-xylose based linkage; formula 11 is a 12 x 6 switchback containing an o-xylose derived linker; formula 12 represents a 15 x 3 switchback with a propylene glycol linker; formula 13 represents a 12 x 6 switchback with a propylene glycol linker. The oligomers were tested for triplex formation using Tm measurements as described in Example 8 above. A control oligonucleotide of the sequence 5'-TTCTTTTTCTTCTTT-3' gave a Tm of 34.1°C. The compounds of formulas 12 and 13 gave comparable or lower Tms; the Tm for formula 12 was 34.2°C, that for formula 13 was 26.5°C. However, the oligomers containing o-xylose type linkages showed a dramatic enhancement in Tm; for formula 10, 44.7°C, and for formula 11, 41.6°C.

A diagrammatic summary of the three triple helix forming strategies is found in Figure 5.

Figure 5A shows the strategy of inverted polarity with constant motif; figure 5B shows the approach of constant polarity and motif switch and figure 5C shows oligomers which bind to a single strand target region (without crossover).

Claims

1. An oligonucleotide comprising

a first nucleotide sequence containing at least three nucleotide residues, said sequence having either 3'→5' or 5'→3' polarity, and, coupled thereto,

a second nucleotide sequence containing at least one nucleotide residue, said second sequence having polarity inverted from that of the first sequence.

2. The oligonucleotide of claim 1 which is capable of forming a triplex with a target duplex DNA.

3. The oligonucleotide of claim 1-2 wherein

the 5' position of a nucleotide at the 5' end of the first sequence is coupled to the 5' position of the nucleotide at the 5' end of the second sequence, or wherein

the 3' position of a nucleotide at the 3' end of the first sequence is coupled to the 3' position of the nucleotide at the 3' end of the second sequence, or wherein

the base of the nucleotide at the 5' end of the first sequence is coupled to the base of the nucleotide at the 5' end of the second sequence, or wherein

the base of the nucleotide at the 3' end of the first sequence is coupled to the base of the nucleotide at the 3' end of the second sequence, or wherein

the base of the nucleotide at the 5' end of the first sequence is coupled to the 5' position of the nucleotide at the 5' end of the second sequence, or wherein

the 5' position of the nucleotide at the 5' end of the first sequence is coupled to the base of the

nucleotide at the 5' end of the second sequence, or wherein

the base of the nucleotide at the 3' end of the first sequence is coupled to the 3' position of the nucleotide at the 3' end of the second sequence, or wherein

the 3' position of the nucleotide at the 3' end of the first sequence is coupled to the base of the nucleotide at the 3' end of the second sequence.

4. The oligonucleotide of claim 1-3 wherein said first and second sequences are coupled through a linkage comprising a linker residue.

5. The oligonucleotide of claim 6 wherein said linker residue is of the formula:

$-(CH_2)_{n1}-$,

wherein n1 is an integer of 1-15 and one or more of said CH_2 groups may be replaced by O, S, or NH, provided said replacement is not adjacent to a heteroatom; or

of the formula

$-(CH_2CH_2O)_{n2}-$,

wherein n2 is an integer of 1-5; or

of the formula

$CH_2(CX_2CX_2)_{n3}CH_2$,

wherein n3 is an integer of 1-7 and each pair of X on adjacent C independently are H or together are a π bond; or

of the formula

$CH_2(CX_2CX_2)_{n4}CH_2(CX_2CX_2)_{n5}CH_2$,

wherein n4 and n5 are integers of 0-7 and wherein the sum of n4 and n5 is not greater than 7 and wherein each pair of X on adjacent C independently are H or together are a π bond.

6. The oligonucleotide of claim 4 wherein said linker residue contains at least one cyclic region.

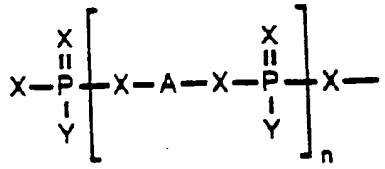
7. The oligonucleotide of claim 6 wherein said cyclic region is nonaromatic.

8. The oligonucleotide of claim 7 wherein said nonaromatic cyclic region comprises a residue of piperidine, piperazine, furan, tetrahydrofuran, cyclohexene, cyclopentene, cyclopentane or cyclohexane.

9. The oligonucleotide of claim 6 wherein said cyclic region is aromatic.

10. The oligonucleotide of claim 9 wherein said aromatic cyclic region is the residue of an o, m, or p disubstituted benzene or disubstituted naphthalene.

11. The oligonucleotide of claim 1-3 wherein said first and second sequence are coupled through a linkage of the formula:



wherein:

Y is H, -OR, -SR, $-NR_2$, O^-, or S^-;

X is O, S, or NR;

wherein each R is independently H, alkyl (1-12C), aryl(6-12C), aralkyl(7-20C) or alkaryl(7-20C);

n is 0 or 1; and

A is the residue of a linker group.

12. The oligonucleotide of claim 11 wherein all X are O and/or all Y are O.

13. The oligonucleotide of claim 1-12 wherein said first and second sequences are enriched in pyrimidine residues.

14. The oligonucleotide of claim 1-13 wherein said first nucleotide sequence is enriched in pyrimidine residues and said second nucleotide sequence is enriched in purine residues.

15. The oligonucleotide of claim 14 wherein said purine residues are guanine residues.

16. An oligonucleotide useful for binding a target DNA duplex to obtain a triplex which oligonucleotide comprises a first nucleotide sequence of at least 3 nucleotide residues enriched in purine residues and a second proximal nucleotide sequence of at least 3 nucleotide residues enriched in pyrimidine residues.

17. The oligonucleotide of claim 16 wherein the purine residues are guanine residues.

18. The oligonucleotide of claim 16 wherein said first and second nucleotide sequence each contain at least five nucleotide residues.

19. A method for binding an oligonucleotide to portions of at least one strand of a target double-helical polynucleotide duplex comprising the step of:

contacting a target double-helical polynucleotide duplex with an oligonucleotide under conditions which permit formation of a triplex;

wherein said oligonucleotide comprises a first sequence of nucleotides capable to bind to a first portion of a strand of said duplex, coupled to a second sequence of nucleotides having inverted polarity from said first sequence capable to bind to a proximal second portion of a strand of said duplex.

20. The method of claim 19 wherein said first portion and said second portion of said duplex are on the same strand in said duplex, and wherein one of said first and second sequences in the oligonucleotide is enriched in pyrimidines and the other is enriched in purines.

21. The method of claim 20 wherein the first and second portions of said duplex are on opposite strands of said duplex and said both of said first and second sequences are enriched in pyrimidines or purines.

22. A method for binding an oligonucleotide to portions of both strands of a target double-helical polynucleotide duplex comprising the step of:

contacting said target double-helical polynucleotide duplex with an oligonucleotide under conditions which permit formation of a triplex;

wherein said oligonucleotide comprises a first sequence of nucleotides capable to bind to a first portion of the first strand of said duplex, said first sequence being enriched in pyrimidines, coupled to a second sequence of nucleotides capable to bind to a

proximal second portion on the second strand of the duplex, said second sequence being enriched in purines.

23. A pharmaceutical composition suitable for use in antisense therapy, which composition contains, as active ingredient, an effective amount of the oligonucleotide of claims 1-18.

24. A method to treat diseases or conditions mediated by the presence of unwanted duplex polynucleotides, which method comprises administering to a subject in need of such treatment an effective amount of the oligonucleotide of claims 1-18 or a pharmaceutical composition thereof.

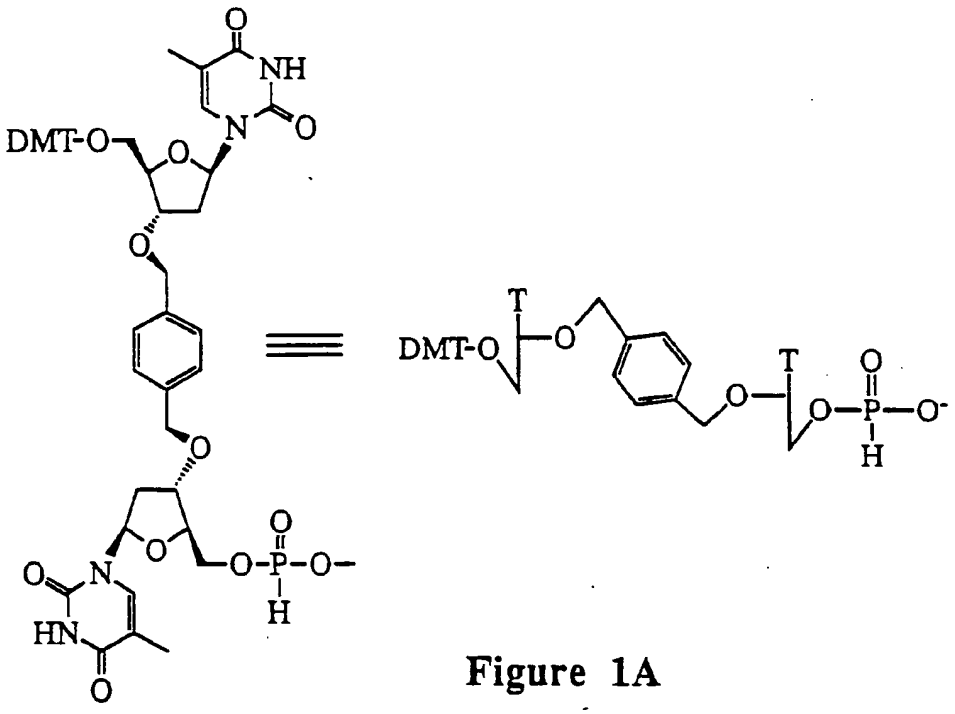


Figure 1A

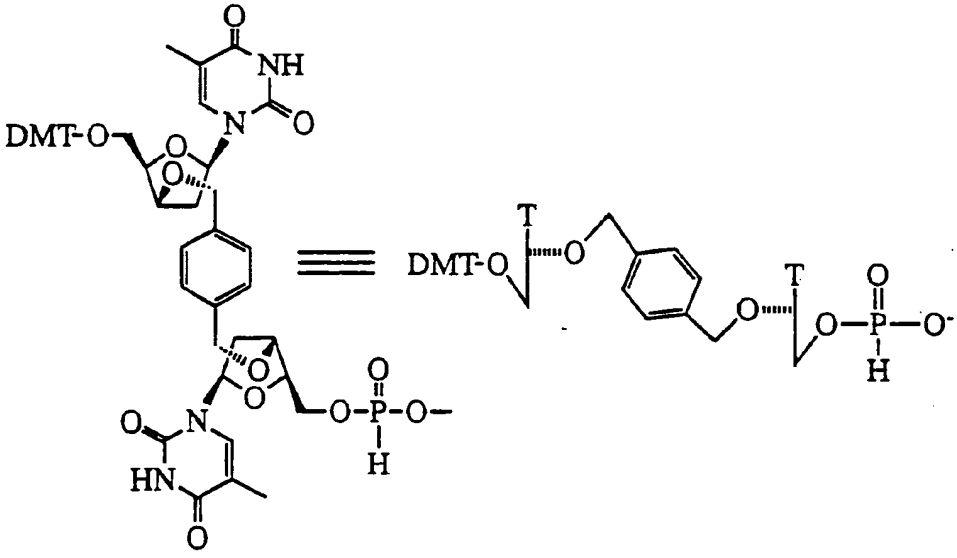


Figure 1B

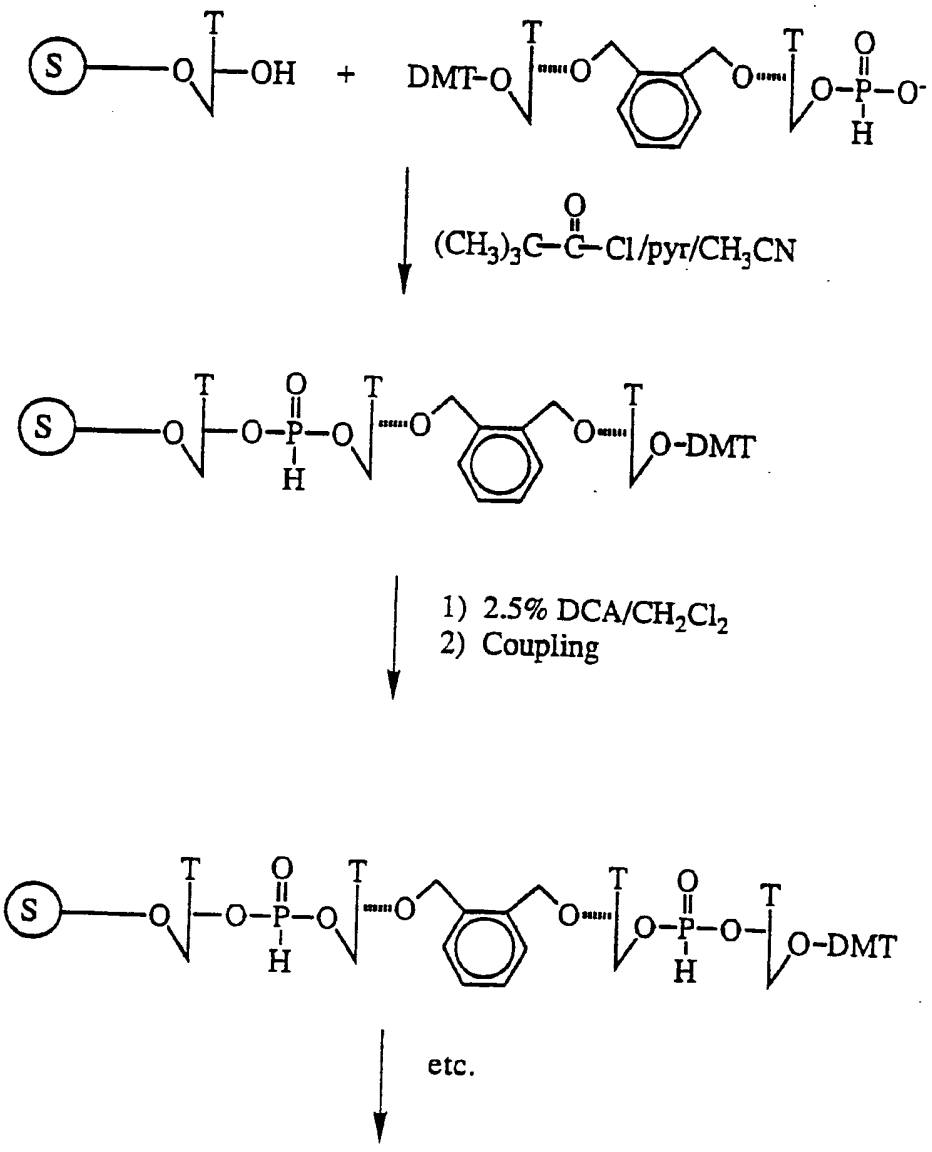

S
T
O
OH
+
DMT-O
O-P-O-
H
(CH3)3C-C-Cl/pyr/CH3CN
O-DMT
1) 2.5% DCA/CH2Cl2
2) Coupling
etc.

Figure 2

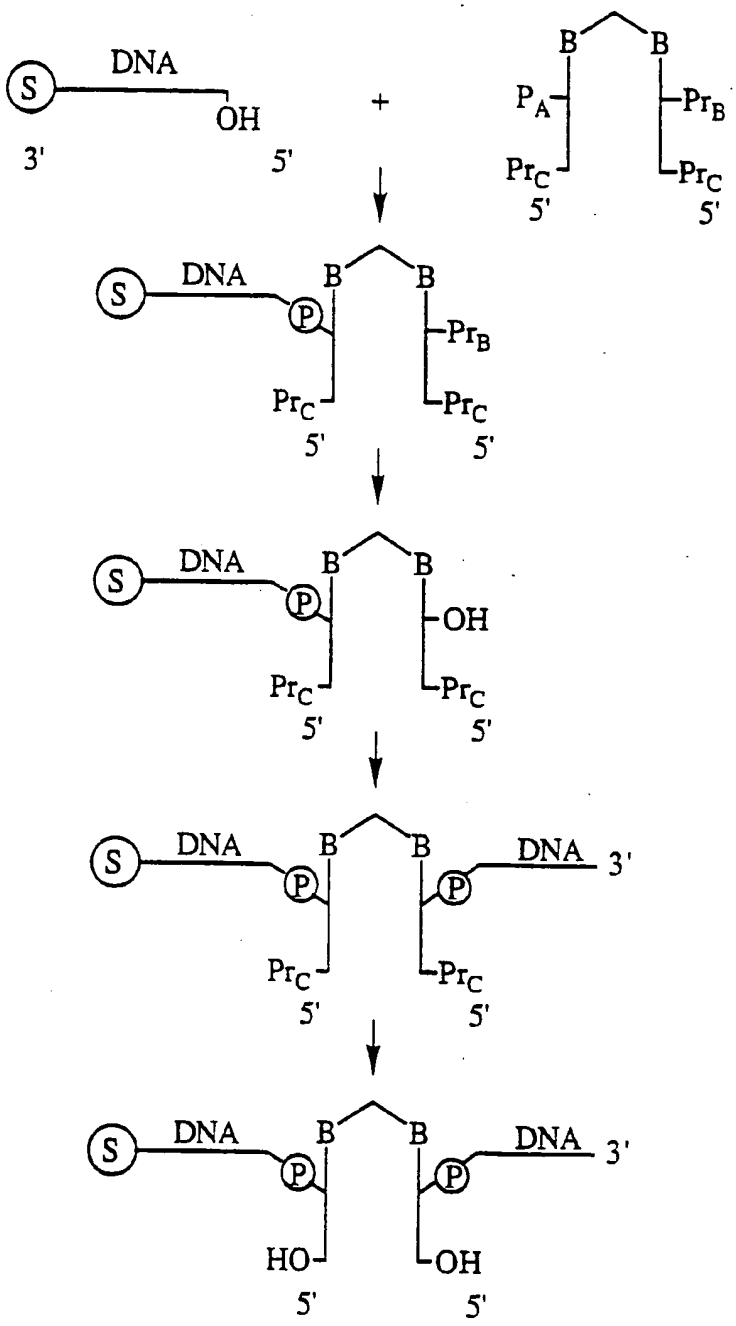


Figure 3

FIGURE 4A

FIGURE 4B

FIGURE 4C

FIGURE 4D

FIGURE 4E

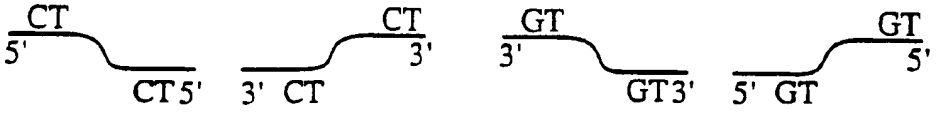


Figure 5A

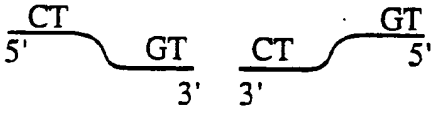


Figure 5B

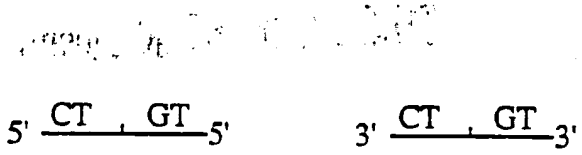


Figure 5C